Anticancer Activity of a Combination of Cisplatin and Fisetin in Embryonal Carcinoma Cells and Xenograft Tumors

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Abstract

Use of chemotherapeutic drug cisplatin is limited because of its toxicity. Therefore, efforts continue for the discovery of novel combination therapies with cisplatin to reduce its effective treatment dose. This study evaluates the potential of fisetin, a flavonoid, to increase cisplatin cytotoxicity in human embryonal carcinoma NT2/D1 cells. Addition of fisetin to cisplatin enhanced cisplatin cytotoxicity in vitro four times lower dose than that required by cisplatin monotherapy for similar cytotoxic effects. Cisplatin, fisetin monotherapy, and addition of fisetin to cisplatin in a combination increased FasL expression. Cisplatin and fisetin as single agents activated caspases-8 and -3 and caspases-9 and -7, respectively, whereas combination treatment activated all 4 caspases. Increases in p53 and p21 and decreases in cyclin B1 and survivin occurred, all effects being more exaggerated with the combination. Fisetin, with or without cisplatin, increased expression of proapoptotic protein Bak and induced its mitochondrial oligomerization. Bid truncation and mitochondrial translocation of Bid and p53 was induced by fisetin in the presence or absence of cisplatin. Downregulation of p53 by short hairpin RNA during drug treatment decreased p21 levels but caused survivin increase, thus reducing cell death. Upstream to p53, inhibition of p38 phosphorylation reduced p53 phosphorylation and cell death. In a NT2/D1 mouse xenograft model, combination therapy was most effective in reducing tumor size. In summary, findings of this study suggest that addition of fisetin to cisplatin activates both the mitochondrial and the cell death receptor pathway and could be a promising regimen for the elimination of embryonal carcinoma cells. Mol Cancer Ther; 10(2); 255–68. ©2011 AACR.

Introduction

Over the last 40 years, incidences of testicular carcinoma have steadily increased, becoming the most common cancer in young men (1, 2). A percentage of these carcinomas originating from germ cells either do not respond to treatment or recur post chemotherapy (2, 3). Testicular teratocarcinomas and embryonal carcinoma cells derived from these tumors are extremely sensitive to cisplatin [cis-diamminedichloroplatinum (II)] and its derivatives (4–6); however, use of high doses of cisplatin is limited by the occurrence of various side effects (7, 8). Combination regimens with cisplatin have been tried with moderate success; however, the search continues for improved formulations (9, 10).

Flavonoids, a group of plant secondary metabolites, show anticancer properties in vitro and in animal models of carcinogenesis of a variety of cancers, and a number of them are undergoing clinical trials (11). Flavonoids genistein, quercetin, silybin, and fisetin are known to augment effects of chemotherapeutic agents (12–14). A combination of phenoxodiol, an analogue of isoflavone genistein (15), together with carboplatin has entered phase-III clinical trials for the treatment of ovarian cancer (http://www.OVATURE-trial.com). The above reports suggest the importance of exploring and improving flavonoid combinations for cancer treatment. Fisetin (3,3',4',7-tetrahydroxyflavone), a flavonoid found in fruits and vegetables, inhibits proliferation of multiple types of cancer cells (16–18) through the inhibition of ERK phosphorylation (18), topoisomerase activation (19), induction of PI3K/Akt, JNK signaling (20), caspase-3, -9, and -7 activation, p53 (16), and NF-κB (14) expression. Because of its ability to affect multiple signaling pathways, fisetin is a suitable candidate to serve as a partner for chemotherapeutic agents for the formulation of novel treatment regimens with higher potency.

The specific goals of this study were (i) to determine the anticancer efficacy of a combination of cisplatin and fisetin on embryonal carcinoma cells in vitro, (ii) to obtain mechanistic insights into the mode of action of the combination, and (iii) to determine if this combination could affect tumor growth in vivo. The results show that when fisetin is combined with cisplatin, cell death induced is much higher...
as compared with monotherapeutic treatments with both agents in vitro and in vivo. Also, the effective dose of cisplatin required to induce significant cell death is reduced when combined with fisetin. Mechanistically, multiple death pathways are activated by the combination that generates the amplified effect on cell death.

Materials and Methods

Materials
Cisplatin [cis-diaminedichloro platinum (II)], Fisetin (3,3’,4’,7-tetrahydroxyflavone, 5-deoxyquercetin), SB 203580, Collagenase type-II DNAse I, and propidium iodide (PI), custom made oligonucleotides and all other chemicals unless mentioned were obtained from Sigma–Aldrich. Antibodies to caspase-8, caspase-3, FasL, Bid, Bak, and caspase inhibitors Z-VAD-fmk, Z-IETD-fmk, Z-DEVd-fmk, Z-LEHD-fmk, and caspase substrates Ac-IETD-AFC, Ac-IETD-CHO, and Matrigel were from BD Biosciences. Mitochondrial hsp-60, anti-phospho p38 (thr 180, tyr 182) and total p38 antibodies were from StressGen Biotechnologies. Antibodies against cyclin B1, PARP, histone, GAPDH, cytochrome C, caspase-9, and p53 were from Santa Cruz Biotechnology and antibodies against caspase-7, p21 waf1/cip1, survivin, and phosphoserine-15-p53 were from Cell Signaling Technology. Anti-p53 antibody for immunofluorescence and anti-actin antibodies came from Roche Diagnostics, whereas all secondary antibodies were from Jackson Immuno Research Laboratory. Terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) kit and CellTiter 96 AQuesous nonradioactive Cell Proliferation Assay kit (MTS assay) were from Promega. Mitotracker Red, Hoechst 33342, JC-1, and EnzCheck caspase-3 assay kit#1 were from Molecular Probes. Negative control and p53 siRNAs were purchased from Ambion and Dharmaco, respectively. Transpass R2 was from New England Biolabs. G Biosciences and Biological Industries supplied CBX protein estimation kit and fetal bovine serum (FBS) and enhanced chemiluminescence system, respectively. FuGENE-6 was purchased from Roche Diagnostics.

Cells
NT2/D1 cells, a pluripotent cell line (kind gift from Dr. M. Inamdar of JNCASR, Bangalore with consent from Dr. P. Andrews, University of Sheffield, U.K.) derived from a human teratocarcinoma were maintained in Dr. M. Inamdar of JNCASR, Bangalore with consent from Cells FuGENE-6 was purchased from Roche Diagnostics.

Caspase activity assay
Activities of caspases-3 and -8 were assayed according to manufacturer’s instructions using the caspase specific fluorescence peptide substrates Ac-DEVd-AMC and Ac-IETD-AFC, respectively. Fluorescence from free 7-amino-4-methyl coumarin (AMC) was monitored in Fluostar Omega (BMG Labtech) using 340 nm excitation and 485 nm emission and AFC (7-amino-4-trifluoromethyl coumarin) using spectrofluorometer (PerkinElmer) with excitation wavelength of 400 nm and emission of 450-550 nm.

SDS-PAGE and Western Blot
Cells were lysed with lysis buffer (0.125 mol/L Tris, 4% SDS, 20% glycerol, and 10% 2-ME). Protein concentrations cultures at different doses mentioned in Results. Treatments for xenograft tumors were given as injections daily for a period of 10 days intraperitoneally at doses mentioned in Results.

Cell viability and measurement of mitochondrial membrane potential
For cell viability, CellTiter 96 AQuesous One Solution Cell Proliferation Assay kit (Promega) was used as per manufacturer's instructions. Briefly, treated cells were incubated with MTS reagent at 37°C (4 hours) and absorbance was measured at 470 nm with a Microplate Reader (μQuant, Bio-tek instruments). Combination index (CI) was derived from cell death indices achieved with cisplatin (0.5–10 μg/mL) plus fisetin (2–40 μmol/L) at a fixed concentration ratio of 1:4. On the basis of an isobologram generated from the above data for mutually exclusive effects, the CI value was calculated as: CI = (D1/D1)1+(D2/D2)2, where (D1)1 and (D2)2 are the concentrations of the single drugs required to induce cell death by 80%, and (D1) and (D2) are the drug concentrations in combination treatments which also induce cell death by 80% (isoeffective as compared with single drugs). CI < 1, CI = 1, and CI > 1 indicate synergism, additive effect, and antagonism, respectively (21). Cells fixed in 70% ice-cold ethanol and stained with PI were analyzed by flow cytometry for detection of membrane permeable cells. MMP was measured using a potentiometric probe JC-1 as described previously (22).

Subcellular fractionation
For subcellular fractionation, cells swelled in hypotonic buffer (10 mmol/L NaCl, 1.5 mmol/L MgCl2, 10 mmol/L Tris-HCl pH 7.5) were homogenized with a Dounce homogenizer (50 strokes) and resuspended in mitochondrial stabilization buffer (525 mmol/L mannitol, 175 mmol/L sucrose, 1.25 mmol/L Tris-HCl pH 7.5, 2.5 mol/L EDTA, pH 7.5). The homogenate was centrifuged at 13,000 g at 4°C for 10 minutes and resuspended in mitochondrial stabilization buffer. The supernatant was centrifuged at 100,000 g at 4°C for 1 hour to obtain the mitochondrial fraction. Postmitochondrial supernatant centrifuged at 100,000 g × 10 minutes in Optima XL-100K (Beckman Coulter) to isolate the mitochondrial fraction. Postmitochondrial supernatant centrifuged at 100,000 g × 10 minutes in Optima XL-100K (Beckman Coulter) to isolate the mitochondrial fraction. Postmitochondrial supernatant centrifuged at 100,000 g × 10 minutes in Optima XL-100K (Beckman Coulter) to isolate the mitochondrial fraction. Postmitochondrial supernatant centrifuged at 100,000 g × 10 minutes in Optima XL-100K (Beckman Coulter) to isolate the mitochondrial fraction.
were determined by the CBX protein assay kit (G-biosciences). Cell lysates (20–50 μg) were electrophoresed in 12% SDS polyacrylamide gels and transferred onto nitrocellulose membranes at room temperature. Blocking was done in 5% nonfat dry milk for all non-phospho antibodies used and 5% BSA for all anti-phospho antibodies in 0.05% PBS-Tween 20. The membranes were incubated first with primary antibodies at required dilutions [anti-caspase-8,-9,-3, cyclin B1, cytochrome C, and Bak (1:1,000), anti-mhspp60, phospho-p53, phospho-p38, total p38, histone, p21 and survivin (1:2,000), anti-p53 (1:4,000), FasL, GAPDH and Bid (1:5,000) and Actin (1:10,000)] in PBS-Tween 20 containing 1% of appropriate blocking reagent and incubated overnight at 4°C. The blots were subsequently incubated with secondary antibody conjugated to horseradish peroxidase at 1:5,000–1:20,000 dilution in 0.05% PBS-Tween 20 for 1 hour at room temperature. Protein bands were visualized on X-ray films using the enhanced chemiluminescence system (23).

Downregulation of p53 using short hairpin RNA and small interfering RNA

We obtained pLKO.1-based short hairpin (sh) constructs specific for p53 (Addgene plasmid 19119) from the plasmid repository at Addgene (24). Plasmids containing a sh construct specific for p53 and vector control were transfected together with the lentiviral packaging (pMDL), envelope (CMV-VSVG), and rev-expressing (RSV-REV) constructs into actively growing HEK293 cells as per protocol provided by Addgene using FuGENE-6 as the transfection reagent. Virus-containing supernatants were collected at 48 hours after transfection and NT2/D1 cells were infected and selected for puromycin resistance and analyzed at 48–72 hours after infection.

Cells were transfected with 40 pmol SMART pool Tp53 or negative control siRNA using Transpass R2 transfection reagent as per manufacturer’s instructions (New England Biolabs). Transfection complex formation was initiated by mixing siRNA with transfection reagent prior to incubating them with 10^5 cells/well/24 well plate for 6 hours. Target protein knockdown was assessed 24 hours post-transfection by probing extracts of cells on Western blots with anti-p53 antibodies.

Histology and immunocytochemistry

Cells were labeled with Mitotracker Red for specific staining of mitochondria prior to fixation in chilled methanol followed by staining with anti-p53 antibody and secondary antibody conjugated to Alexa-fluor 488 (Jackson Immuno Research Laboratory). An inverted microscope [(TE-2000E) Nikon] equipped with a RetigaExi camera (Q-imaging) was used to acquire images and coefficient of colocalization was calculated from mask of colocalization using Image-Pro Plus software (Media Cybernetics). For TUNEL labeling, procedure was carried out as described previously (25).

After euthanasia, animals were dissected for removal of tumors and various other organs for fixation in 4% formaldehyde and Bouin’s fluid for TUNEL staining and routine histology, respectively. Tissues were processed as per standard protocol (26).

Xenograft tumors

All experimental procedures were done according to the standards specified by Institutional Animal Ethics Committee of National Institute of Immunology, New Delhi. NT2/D1 cells (10^6) mixed with Matrigel were implanted into 6-week-old athymic nude mice bearing the nu/nu gene [NIH(s) (nu/nu)] and housed under pathogen-free conditions. The tumor (300–350 mm^3) bearing mice were treated with cisplatin (2.5 mg/kg), fisetin (2 mg/kg), and combination of both the drugs, in which individual dosage was (cisplatin, 1.5 mg/kg + fisetin, 1 mg/kg) intraperitoneally per day for 10 days. Another group of animals were treated with monotherapy of cisplatin (1.5 mg/kg) and fisetin (1 mg/kg). Controls were treated with vehicle only. Xenografted tumor volume was measured using Vernier callipers (major and minor axis) and tumor volume was calculated by the equation: L × W^2/2 (mm^3), where L = length and W = width.

Statistical analysis

Data were analyzed by student’s unpaired t-test. The values were considered significantly different at P < 0.05. All experiments were repeated 3–5 times and data expressed as the mean ± SE of several independent experiments.

Results

Cytotoxic activity of cisplatin and fisetin is enhanced when combined together

To determine the cytotoxic effects of cisplatin when given along with fisetin, NT2/D1 cells were exposed to cisplatin and fisetin (Fig. 1A) as monotherapies as well as in combination. Moderate cytotoxic activity with both compounds as monotherapy was evident with a dose-dependent reduction in cell number obtained using the MTS assay (dosage range cisplatin, 0.5–100 μg; fisetin, 2-100 μmol/L; Fig. 1B). A combination of both drugs at a fixed ratio (1:4) over a dosage range was tested for possible potentiation of cisplatin effects by fisetin (Fig. 1B). These data were used to determine CI values through an isobologram (21) calculated as mentioned in Materials and Methods. Combination doses from 2 μg/ml cisplatin and 8 μmol/L of fisetin to 4 μg/ml cisplatin and 16 μmol/L of fisetin fell within the envelope of nearly additive effects (Fig. 1B). A CI value of 1 or less (0.65) was obtained with 5 μg/ml of cisplatin and 20 μmol/L of fisetin (Fig. 1B), indicating that the interaction between cisplatin and fisetin was synergistic at this dose. This particular combination dose was used for further studies on mechanistic aspects.
Cisplatin, fisetin and the combination induces apoptotic death

We next tested if the cytotoxicity observed was due to cellular apoptosis. Figure 1C shows a significant increase in sub-G₁ cell population representing formation of apoptotic bodies (27) with the combination treatment over a 10-hour-period. Monotherapy with either cisplatin or fisetin showed approximately 36% lower accumulation of sub-G₁ cells suggesting higher rate of apoptosis when fisetin was added to cisplatin. To dissect the mechanism for cell death, cells pretreated with caspase inhibitors were exposed to the drugs to assess if cell death was caspase-dependent. Z-VAD-fmk, a pan caspase inhibitor, blocked cell death in all groups (Fig. 1D, c, h, m) suggesting caspase involvement with all treatments. Having established that induction of cell death was caspase-dependent, efforts were made to identify specific caspases induced by different treatments with the use of inhibitors specific for particular caspases. Inhibition of caspase-3 only with Z-DEVD-fmk blocked death in cisplatin (Fig. 1D, f) and the combination treated cells (Fig. 1D, p) but not in fisetin treated cells (Fig. 1D, k). In contrast, Z-LEHD-fmk, a caspase-9 inhibitor, was...
most efficient in reducing cell death with fisetin only (Fig. 1D, j) and the combination treatment (Fig. 1D, o). Z-IETD-fmk, the caspase-8 inhibitor could not reduce cell death in fisetin treated cells but was effective in cisplatin (d) and the combination treated cells (Fig. 1D, n). Further confirmation of specific caspase involvement was determined by monitoring caspase cleavage and activity. Significant cleavage of procaspase-3 as seen on Western blots and increased cleavage of fluorescent caspase-3 substrate by lysates from cisplatin and the combination treated cells further confirmed caspase-3 participation in these two treatment groups (Fig. 2A). Procaspase-9 proteolysis was most prominent with fisetin and the combination treatment at 8 hours (Fig. 2B), which is in concurrence with the ability of caspase-9 inhibitor to block cell death in these two groups shown earlier in Figure 1D. Proteolysis of procaspase-8 to the active 23 kDa fragment was detected in cell lysates from only cisplatin and the combination treatment (Fig. 2C). Fisetin could induce only partial proteolysis of procaspase-8 to the 40/36 kDa form, whereas the combination could generate both the 40/36 kDa and the 23 kDa form (Fig. 2C). These observations were supported by higher caspase-8 activity obtained with cell lysates from cisplatin and the combination treated cells using Ac-IETD-CHO as a caspase-8 substrate (Fig. 2C). These data suggest that the increase in antiproliferative activity observed with the combination of cisplatin and fisetin is attributable at least in part to significant cellular apoptosis mediated by caspases.

The role of FasL in the activation of cell death receptor pathway is well established (28). Increased expression of FasL (Fig. 2D) verified activation of the death receptor pathway in all groups. Because complete proteolysis of procaspase-8 occurred with cisplatin but not with fisetin, signals downstream to caspase-8 proteolysis were investigated. The partial cleavage of caspase-8 to 40/36 kDa fragment in combination and fisetin treated cell lysates prompted us to check for the truncation of Bid, as Bid truncation is known to be induced by incompletely cleaved caspase-8 (29). Truncated Bid was visible in cell lysates from fisetin and the combination treated group with more complete cleavage occurring upon combination treatment (Fig. 2D). Procaspase-7, an executioner caspase that can be activated by caspase-9 (28) was cleaved at 6 hours to its active form in the combination group and at 10 hours in the fisetin treated group (Fig. 2D), suggesting faster activation with the combination. The lack of visibility of cleavage product with the combination treatment at 10 hours could be due to death of a large number of cells by then and the procaspase-7 band visible probably originates from remaining surviving cells. The cleavage of caspase-7 was caspase-9 dependent as caspase-9 inhibitor Z-LEHD-fmk blocked caspase-7 cleavage when present during drug treatment (Supplementary Fig. S1). In summary, participation of the death receptor pathway in execution of apoptosis was evident in all groups with cisplatin and fisetin using different initiator and executioner caspases. Addition of fisetin to cisplatin induced caspases-8, -3, -9, and -7 in tandem, possibly inducing amplification of effects with the combination.

**Cisplatin and fisetin combination induces p53 mediated death pathway**

The tumor suppressor protein p53 coordinates a complex network of cellular proteins evolved to protect cells from malignant transformation and can play a proapoptotic role (30). Unlike many other malignant cells, NT2/D1 expresses an active p53 (31). The p53 expression increased with all 3 treatments but this increase was most significant with fisetin and the combination treatment (Fig. 3A). The downstream transcriptional target for p53, p21 protein levels also increased in a similar fashion as p53 (Fig. 3A). Cyclin B1, a regulatory protein involved in mitosis (32) was downregulated to a greater degree in the combination treated cell lysates as compared with fisetin, whereas cisplatin did not induce any significant change (Fig. 3A). Survivin, an inhibitor of apoptosis protein (32) proteins was significantly downregulated with the combination treatment as compared with monotherapeutic administrations (Fig. 3A). Subcellular localization of p53 is indicative of its function. Investigations into the p53 subcellular localization showed heavy nuclear concentration of the protein with the combination treatment but cisplatin and fisetin monotherapy did not induce any significant change (Fig. 3B; Supplementary Fig. S2). Mitochondrial localization of p53 where it can act as "BH3 only protein" is known (33). With our studies, it is evident that fisetin treatment with or without cisplatin induced higher p53 concentration in the mitochondria (Fig. 3B; Supplementary Fig. S3, S4, S5 vehicle control). In contrast to fisetin, cisplatin treatment did not induce any mitochondrial accumulation of p53 (Fig. 3B; Supplementary Fig. S2). Post-translational modifications like phosphorylation induce protein activity (34). Phosphorylation of p53 is predicted to be essential for its stabilization, activation of target genes, and induction of apoptosis (35). Phosphorylation at Ser-15 is linked to cellular apoptosis (35) and in NT2/D1 cells, phosphorylation occurred at Ser-15 position (Fig. 3C) with the combination treatment suggesting proapoptotic role of p53 in NT2/D1 apoptosis.

The changes in p53 described above suggested a major role of the molecule in mediating the effects of cisplatin and fisetin on cell death. This prompted us to investigate cellular response in the absence of p53. A stable cell line was generated through the expression of p53 shRNA, where cellular expression of p53 was significantly lower as compared with non-transfected cells (Fig. 3D). As a consequence of p53 downregulation, a prominent decrease in p21 levels occurred in all 3 groups with concomitant upregulation of survivin (Fig. 3D). As shown in Figure 3D, the treatments could induce lower cell death under p53 knockdown conditions as compared with cells with normal constitutive p53 expression. These biochemical alterations are reflected in morphological changes. Our studies show a distinct difference in cellular morphology...
Figure 2. Caspase activity during drug treatments. A, Western blot showing the processing of procaspase-3 at 10 hours with cisplatin, fisetin, and cisplatin + fisetin treatment. Actin blot shows the loading control. Caspase-3 activity plot shows measurement of caspase-3 activity using Ac-DEVD-AMC as substrate with cell lysates collected at 8 hours post-treatment. Data represent ± SE (n = 3). Pro c-3, procaspase-3; casp-3, caspase-3. B, Western blot showing the processing of procaspase-9 at 8 hours. Bar graph represents densitometric measurements of procaspase-9 processing on the Western blot. Data are normalized with actin immunoreactivity used as loading controls. Pro c-9, procaspase-9. Data are mean ± SE (n = 3). C, Western blot of cell extracts from cisplatin, fisetin, and the combination of cisplatin and fisetin treated cells probed with anti-caspase-8 antibody showing cleavage of procaspase-8 to 46/36 kDa and 23 kDa fragments. Note that fisetin treated cells shows proteolysis of procaspase-8 to 46/36 kDa but not to 23 kDa active fragments. Measurement of caspase-8 activity using Ac-IETD-AFC as substrate shows caspase-8 activity obtained with cell lysates from different treatment groups at 8 hours after treatment. Data are representative of a minimum of 3 experiments. Pro c-8, procaspase-8; C, cisplatin; F, fisetin; C+F, cisplatin + fisetin; C+F+I, cisplatin + fisetin + caspase inhibitor. D, Western blots of cell extracts probed with anti-FasL antibody shows increase of FasL expression as compared with vehicle treated controls in all 3 groups of treatment at 6 and 8 hours. Bars show relative expression of FasL measured by densitometry and normalized to actin immunoreactivity used as loading controls. Western blots of cell extracts probed with anti-Bid antibody showing the cleavage of Bid with different treatments. Immunoblot analysis for caspase-7 at 6 hours and 10 hours show cleavage of caspase-7 to its active form. Data are mean ± SE (n = 3). Pro c-7, procaspase-7; Cis, cisplatin; Fis, fisetin; F, fisetin; Cis + Fis, cisplatin + fisetin.
between the drug treated cells in the vector controls versus the p53 knockdown cells (Fig. 3D, cell morphology). Detachment of cells from the substratum and rounding up were the prominent features of treated cells transfected with vector only, whereas cells under p53 knockdown conditions show adherent cells with clear spread out morphology and lesser detachment from the substratum. Knockdown of p53 using siRNA showed similar results with the different treatments (Supplementary Fig. S6).

Mitochondria is involved in fisetin mediated cell death
The truncation of Bid suggested mitochondrial involvement in drug-induced cellular apoptosis and one of the established methods to assess mitochondrial function is measuring the loss of mitochondrial membrane potential (36). We observed a significant loss of potential in the combination as well as the fisetin treated cells (Fig. 4A). Mitochondrial potential decrease results in cytochrome c release to the cytosol (36). Cytochrome c release was observed with both fisetin and the combination treatment (Fig. 4B), suggesting mitochondrial involvement between these two treatment models. Other than Bid translocation to the mitochondria, pro-apoptotic proteins like Bak could be involved in the release of cytochrome c as well (36). Significant up-regulation of Bak along with its oligomerization in the mitochondria was observed most prominently with the combination (Fig. 4C). PARP cleavage (28), which is a hallmark of caspase-3 and -7 activation was visible with all 3 groups with the combination inducing significant cleavage by 10 hours (Fig. 4D).

Cisplatin and fisetin combination increases phosphorylation of p38
Prior knowledge that p38 regulates events upstream to p53 (37) prompted us to investigate possible changes in p38. No change was observed in total expression levels of p38 but increased phosphorylation (Thr 180/Tyr182) occurred with all treatments (Fig. 5A). Because phosphorylation regulates activity of proteins, phosphorylation inhibitor for p38, SB203580 (37) was used to inhibit p38 phosphorylation (Fig. 5A). As a consequence of p38 phosphorylation inhibition, there was a reduction in p38 phosphorylation as well (Fig. 5B). Biological parameters like mitochondrial potential decrease, as a consequence of drug treatments, was inhibited by SB203580 (Fig. 5C). Changes in potential were markedly induced by cisplatin and fisetin together that could be inhibited by SB203580. This inhibition translated into the rescue of cells from death in all groups being most significant with the combination. These observations suggested involvement of p38 in p53 mediated effects (Fig. 5D).

Combination of cisplatin and fisetin inhibits growth of xenograft tumor in mice
To examine if our studies in vitro were reproducible in a model of NT2/D1 xenograft, tumors were induced in athymic nude mice. Monotherapeutic doses of cisplatin (2.5 mg/kg/day) and fisetin (2 mg/kg/day) showed significant regression of tumors (Fig. 6A and B; Supplementary Fig. S7). Addition of fisetin to cisplatin at the same dose as above was toxic and the mice did not survive beyond 5 days of treatment. However, addition of a lower dose of fisetin (1 mg/kg/day) to cisplatin (1.5 mg/kg/day) was not cytotoxic but showed significant reduction of tumor size over a period of 10 days (Fig. 6A and B; Supplementary Fig. S7). The extent of tumor regression achieved with this treatment was significantly more than the monotherapeutic treatments (Fig. 6A and B). There was no significant reduction in body weight post treatment except in the control or the cisplatin treated group (Fig. 6C). To determine the extent of cellular apoptosis within the tumors, tissue sections were prepared for TUNEL staining. As shown in Figure 6D, TUNEL positive apoptotic cells were detectable in tumor sections from monotherapeutic treatments of cisplatin and fisetin but not in vehicle-treated tumors (Fig. 6D). However, a large population of TUNEL positive cells as shown by cells with brown nuclear deposits was substantially higher in the combination group on day 5 of treatment when cisplatin was administered along with fisetin (Fig. 6D). Sections from 5-day-old tumors are presented in the figures as these tumors were of reasonable size for obtaining a large enough cell population (Fig. 6D).

For checking the cytotoxic potential of the different treatment schedules, histological sections from the kidney of treated animals were stained for apoptotic cells. Very few TUNEL positive cells were visible in the kidney of animals receiving monotherapeutic treatments with cisplatin (2.5 mg/kg/day), fisetin (2 mg/kg/day), and the combination (cisplatin, 1.5 mg/kg/day + fisetin, 1 mg/kg/day; Supplementary Fig. S8). However, a higher number of TUNEL positive cells were detected in the kidney cortex of animals treated with the combination of both drugs at a higher dose (cisplatin, 2.5 mg/kg/day + fisetin, 2 mg/kg/day) on day 4 of treatment (Supplementary Fig. S8). Cellular morphology did not show any marked change in liver, spleen, and kidney of the monotherapies and the combination group (cisplatin, 1.5 mg/kg/day + fisetin, 1 mg/kg/day; Supplementary Fig. S9A–C).

Discussion
NT2/D1 teratocarcinoma cells in vitro and a xenograft model were used to investigate a potential strategy for increasing the effectiveness of cisplatin in killing teratocarcinoma cells by combining the flavonoid fisetin with cisplatin. Cisplatin is one of the most widely used chemotherapeutic drug (38, 39) and fisetin is a flavonoid known to eliminate cancer cells (14). Recently, fisetin’s role as a potentiation factor in killing cancer cells has been shown by its augmentative actions on the induction of cell death by radiotherapy in colorectal cancer cells and with cisplatin in Burkitt’s lymphoma cells (14, 40). How-
ever, the mechanism by which fisetin contributes to the increase in cytotoxic activities of such treatments is not known. Our findings from in vitro and in vivo studies support the hypothesis that combining fisetin with cisplatin potentiates cisplatin’s ability to induce apoptosis of NT2/D1 cells and provoke NT2/D1 xenograft tumor regression. The clinical implications of these laboratory findings suggest that: (a) for a fixed dose of cisplatin, addition of fisetin may improve antitumor responses; (b) cisplatin may be given at a reduced and potentially less toxic dose in combination with fisetin; and (c) components of the involved apoptotic pathways identified could be manipulated to achieve further improvement in treatment modalities.
Our observations on cisplatin-induced death of NT2/D1 cells both in vitro and in vivo is consistent with the ability of testicular teratocarcinomas to respond favorably to treatment with cisplatin (41, 42). Sensitivity of embryonal carcinoma cells to fisetin only treatment observed in these studies add to observations on fisetin induced death of prostate, pancreatic, colon, and hepatocellular carcinoma cells (16, 17, 43, 44). Adding fisetin with cisplatin as a combination to NT2/D1 cells resulted in significantly greater cell death with a CI value of 0.65 suggesting synergism (45, 46), clearly establishing fisetin’s ability to potentiate cisplatin effects. Interestingly, in prostate cancer PC3 cells, autophagic death is induced by fisetin through suppression of mTOR (47), an observation not reproduced in NT2/D1 cells at the dose used (data not shown).

The observations of increased expression of FasL and procaspase-8 proteolysis (28) with all 3 treatments clearly indicated activation of caspase-dependent death pathway through death receptor signaling. However, downstream to procaspase-8 proteolysis, alternate signaling pathways were engaged by cisplatin and fisetin monotherapy because unlike cisplatin, fisetin induced only partial proteolysis of procaspase-8 that is favorably associated with Bid truncation (29). This was confirmed by clear Bid truncation with both fisetin monotherapy and the combination suggesting activation of mitochondrial death pathway in these 2 treatment models (36). Proapoptotic proteins of the Bcl-2 family induce mitochondrial disruption in terms of loss of mitochondrial potential and cytochrome c release (36). In our experiments, largely, 2 proapoptotic mitochondrial proteins, Bid and Bak were involved in the activation of the mitochondrial death pathway with fisetin and the combination treatment resulting in signal amplification. Interestingly, fisetin by itself elicits similar events in human colon and prostate cancer cells (16, 43). The concomitant translocation of p53 to the mitochondria in 2 groups where Bid and Bak were active suggested that p53 could act as a proapoptotic "BH3 only" protein because Bak is known to be "freed" by p53 on the mitochondrial membrane to be able to form oligomers (30). The p53 protein is a tumor suppressor, which acts as a checkpoint in cell cycle either preventing or initiating programmed cell death (48) and is mutated in many forms of cancer (48). However, in teratocarcinoma cells, this rate of mutation is very low (41) and therefore, p53 has an important role as observed in this study.

In NT2/D1 cells, fisetin primarily used caspase-7 as executioner caspase instead of caspase-3, which could potentially be activated through the mitochondrial death pathway as in hepatocellular carcinoma where caspase-3 is activated by fisetin directly (17). Direct activation of caspase-3 by cisplatin without mitochondrial involvement is in contrast to observations in ovarian cancer cells (46) HEp-2 cells (49) and osteosarcoma cells (50), where cisplatin is reported to induce death through the mitochondrial pathway. However, direct activation of caspase-3 by cisplatin in other cell types is well known (31). Therefore, apart from the disparity in the use of initiator caspases by cisplatin and fisetin, our studies show the use of distinct executioner caspases as well. Fisetin treatment primarily induced caspase-7 activation through procaspase-9 which is similar to observations with HT-116 colon cancer cells (16, 43) and SK-HEP-1 hepatocellular carcinoma cells (16, 17). In
Figure 4. Changes associated with mitochondria and PARP cleavage. A, flow cytometric analysis of treated and untreated cells stained with potentiometric probe JC-1 for mitochondrial potential measurement. Cis, cisplatin; fis, fisetin; cis + fis, cisplatin + fisetin. The upper quadrant and lower quadrant shows cells stained with JC-1, red staining (FL-2) indicating cells with higher mitochondrial potential and green staining (FL-1) indicating cells with lower mitochondrial potential. Data are representative of 4 experiments. B, Western blots of subcellular fractions of treated and untreated cells probed with anti-cytochrome c antibody showing cytochrome c content in the cytosol and the mitochondria. VT, vehicle control; m, mitochondria; c, cytosol. Antibody against GAPDH was used for the fraction purity. Data are representative of 3-4 experiments. C, Western blots showing expression and oligomerization of Bak in whole cell and mitochondrial lysates, respectively. Relative Bak expression is normalized against actin used as loading control. Data are representative of 3 experiments. Arrows on oligomerization blot indicate oligomerized products. Cytochrome c levels in the mitochondria are shown below. D, Western blots probed with anti-PARP antibody shows PARP cleavage with cisplatin, fisetin, and cisplatin + fisetin. Cis, cisplatin; Fis, fisetin, Cis + Fis, cisplatin + fisetin. Data are representative of 3 experiments.
addition to mitochondrial translocation, a prominent translocation of p53 to the nucleus (51) suggested enhanced transcriptional activity possibly resulting in the upregulation of p21, a cyclin-dependent kinase inhibitor. Previously published results show that p21 regulates expression of survivin, a member of inhibitor to apoptosis protein family (32) in multiple cell types, including hepatocellular carcinoma HepG2 cells (52). Survivin downregulation during drug treatment is in agreement with the increased cell death observed. Deregulation of cyclin B1, which could act as a caspase inhibitor and is connected to neoplastic transformation, is suggested to be an attractive strategy for antiproliferative therapy (53). As seen with survivin, the reduced expres-

Figure 5. Phosphorylation of p38.
A, Western blots of treated cell lysates showing inhibition of p38 phosphorylation with SB 203580. Relative phospho-p38 shown is a value normalized against total p38. Actin was used as a loading control. Data are mean ± SE (n = 3). B, Western blots of total cell lysates probed for p53 phosphorylation (ser-15) shows inhibition of combination induced phosphorylation by SB 203580. Relative p53 expressions are values normalized against actin and relative p53 phosphorylation are values normalized against total p53. Data are mean ± SE (n = 3). C, figure shows recovery of mitochondrial potential fall in the presence of SB 203580 primarily with the fisetin and the combination treatment. Data are representative of at least 4 experiments. D, bar graph shows cell death induced by the various treatments in the presence or absence of p38 phosphorylation inhibitor SB 203580. Data are mean ± SE (n = 3).
Expression of cyclin B1 was concurrent with increased cell death observed. Inhibition of p38 phosphorylation resulted in a decrease of p53 phosphorylation suggesting p38-mediated regulation of p53 activity. In addition, inhibition of p38 phosphorylation also led to the rescue of mitochondrial potential and cell death, suggesting the connectivity between p38, p53 phosphorylation, and cell survival. Our observations of p53 phosphorylation at...
Ser-15 (35) and ensuing decrease in cell death following inhibition of this phosphorylation reiterate the role of p53 as a proapoptotic modulator.

In agreement with the in vitro data, the NT2/D1 xenograft model showed more efficient tumor regression with the combination therapy as compared with the monotherapies. This was consistent with histological observations, in which the number of TUNEL positive cells (54) in tumors treated with the combination far exceeded cells observed with only cisplatin or fisetin treatment. At higher doses of the combination, however, toxicity was detected. Histopathology of the kidney showed no significant changes at the lower dose.

Interpretation of these results in terms of mechanistic implications suggest cooperation of multiple pathways in eliciting death signals through cross-talk between p53 and proapoptotic Bid and Bak with activation of upstream sensors like p38 when fisetin was administered with cisplatin. The recruitment of dual death pathways using multiple caspases adds an additional armamentarium for treatment, with possibilities of enhancement of effect through modulation of individual pathway components. Therefore, this study highlights a new possibility of using a combination therapy of cisplatin with a flavonoid fisetin for elimination of stem teratocarcinoma.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

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